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(54) Title: PROTEIN COUPLING TO LIPID VESICLES

(57) Abstract

A new method of coupling proteins and other targeting molecules such as immunoglobulins to lipid vesicles has been developed. A bifunctional agent forms a covalent bond (esp. an S-S bond) without damaging the lipid structure and permits retention of protein activity. The lipid vesicles may have a diagnostic agent such as an immunodiagnostic agent, a visualization agent, a high density particle or a magnetic particle encapsulated therein.

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# PROTEIN COUPLING TO LIPID VESICLES

## Reference to Related Applications

The present invention is a continuation-in-part of United States Patent Application Serial No. 157,571, filed March 8, 1988, which is a continuation-in-part of United States Patent Application Serial No. 025,525, filed March 13, 1987; United States Patent Application Serial No. 078,658, filed July 28, 1987, and United States Patent Application Serial No. 124,824, filed November 24, 1987.

## Background of the Invention

The present invention relates to the coupling of targeting molecules to lipid vesicles. More particularly, methods of coupling targeting molecules, e.g., proteins, to the lipid bilayers of lipid vesicles or liposomes are disclosed which allow a substantial number of targeting molecules to be coupled to each vesicle without decreasing the stability of the vesicle bilayers. Products made using these methods, including targeted vesicles, are also disclosed.

Almost since their discovery, the possible use of lipid vesicles, primarily the phospholipid lipid vesicles commonly known as liposomes, as agents for carrying materials to specific cell groups or

organs in the body has been discussed. In order to accomplish this, targeting molecules must be attached to the surface of the vesicle. These targeting molecules, when reacted with, or embedded in, the outer surface of the lipid vesicles, cause a reaction between the targeting molecules and a desired feature of the targeted cell group, e.g., a cell surface receptor or an antigenic feature on a cell. Currently used methods of coupling phospholipid vesicles to proteins rely primarily on reacting a bifunctional reagent with the amino group of phosphatidylethanolamine included in the bilayer and coupling the protein to the other end of the bifunctional molecule in the lipid layer. However, this procedure leads to many problems including breakdown of the lipid layer and resulting instability of the lipid vesicles. Another approach has been to couple targeting proteins to palmitic acid chains in the bilayer. However, only a few targeting molecules can be introduced in this way and the same complications arise. Accordingly, only small quantities of targeted molecules have been able to be attached to the vesicle without vesicle breakdown.

Similar coupling procedures can be used for <u>in vitro</u> diagnostic agents, particularly immunodiagnostics, as are used for targeting vesicles. As expected, the same type of problems have been observed concerning vesicle stability.

Accordingly, an object of the invention is to provide a method of coupling proteins or other

targeting molecules to lipid vesicles which providing high levels of coupling while reducing vesicle breakdown.

A further object of the invention is to provide a method of coupling targeting molecules to lipid vesicles which can be used with both phospholipid and nonphospholipid vesicles.

Another object of the invention is to provide a method of coupling proteins to lipid vesicles which does not cause a loss of activity of the protein function.

These and other objects and features of the invention will be apparent from the following description and the claims.

Summary of the Invention

The present invention features a method of coupling proteins and other targeting molecules to lipid vesicles at high frequency while retaining their chemical activity. The method can be used with any lipid vesicle which contains a steroid such as cholesterol as one of its structural components.

Lipid vesicles useful in the methods of the invention have steroids with a free sulfhydryl or SH group incorporated as one of the structural molecules of the lipid phase. This yields a vesicle with free SH groups. The preferred steroid is thiocholesterol which is functionally very similar to cholesterol and

can be introduced into the bilayer at more than 20% molar ratio.

The protein or other targeting molecule to be coupled to the vesicle is modified by reaction with a bifunctional agent which reacts with a free $NH_2$ group on the targeting molecule and provides a free sulfhydryl group available for attachment to the vesicle. The modified targeting molecule, which retains its chemical activity after the modification, is then reacted with the lipid vesicle containing the free sulfhydryl group under conditions such that a S-S bond is formed, thereby covalently linking the targeting molecule to the vesicle. Preferred bifunctional agents are selected from a group consisting of N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate, derivatives and chemical analogs thereof.

Proteins are the targeting molecule of choice for use in the invention. Preferred proteins are lectins, particularly those selected from a group consisting of concanavalin A, wheat germ agglutinin, derivatives, and chemical analogs thereof, and immunoglobulins, particularly monoclonal antibodies and other IgG's and IgM's. Multiple copies of the bifunctional agent can be used on a single protein; in fact, modification of one to ten amino groups per protein is preferred. This is easily accomplished with the bifunctional groups disclosed herein. Other targeting molecules useful in the invention include the peptide hormones.

Although any lipid vesicles could be used in the method of the invention, nonphospholipid vesicles, particularly nonphospholipid paucilamellar lipid vesicles, are preferred. These vesicles may be made from a broad spectrum of materials including polyoxyethylene fatty esters, polyoxyethylene fatty acid ethers, diethanolamines, long-chain acyl amino acid amides, long-chain acyl amides, polyoxyethylene sorbitan mono and tristearates and oleates, polyoxyethylene glyceryl monostearates and monooleates, and glyceryl monostearates and monooleates. In addition to the lipid, steroids such as cholesterol and thiocholesterol are included as structural units and charge-producing materials may also be added.

The invention further features a number of uses of the targeting molecule-vesicle combination. One such use is a method of delivering an encapsulated material to a particular location in a human or animal by encapsulating the material within the lipid vesicle, modifying a proteinaceous targeting molecule such as a immunoglobulin, e.g., a monoclonal or chimeric antibody, or a portion or fragment thereof, with a bifunctional agent that react with free $NH_2$ groups on the protein and reacts with free sulfhydryl groups on the lipid vesicle, reacting the modified targeting molecule and the lipid vesicle so that one or more targeting molecules are on the surface of the lipid vesicle, introducing the targeted lipid vesicle into the animal or human subject, and allowing the targeting molecule to react with the specified target, thereby bringing the

encapsulated material to the specified location. Preferred lipid vesicles for this use are nonphospholipid paucilamellar lipid vesicles as previously described because of their large carrying capacity for encapsulated material.

Another advantageous use is for in vitro and in vivo diagnostics and test materials, particularly as immunodiagnostic agents. Visualization agents, magnetic particles, high density particles, or other materials which permit vesicle separation are encapsulated and a receptor or other reactive protein such as an immunoglobulin is bound to the surface of the lipid vesicle. The vesicles are then used in standard immunological or other receptor ligand reactions as visualization or separation agents.

The invention and its features will be further explained by the following detailed description.

## Detailed Description of Preferred Embodiments

The method of the invention can be used to couple substantially any targeting molecule with free $NH_2$ groups to a lipid vesicle in which thiocholesterol can replace steroids such as cholesterol as a structural component. Proteins are preferred since there are normally a number of free amino groups on the protein which are not involved in its biological activity. These free $NH_2$ groups can be used to react with the bifunctional agent while allowing the protein to retain its normal activity.

The present procedure is particularly useful with lectins such as concanavalin A or wheat germ agglutinin, or immunoglobulins such as IgG. If the immunoglobulins such as monoclonal or chimeric antibodies, or portions or fragments thereof, are used, these make particularly good targeting molecules allowing delivery of the lipid vesicle, including any encapsulated material, to a specified cell type or as an immunodiagnostic tool. Other targeting molecules having free $NH_2$ groups can also be used.

Although any type of lipid vesicle may be used, nonphospholipid paucilamellar lipid vesicles are preferred. These type of vesicles, and their methods of manufacture, are described in detail in co-pending United States Patent Application Serial No. 157,571. In addition, the materials and methods described in co-pending United States Patent Application Serial No. 124,824 can be used. Nonphospholipid materials useful in the present invention include surfactants selected from a group consisting of polyoxyethylene fatty esters having the formula

$$R_1\text{-}COO(C_2H_4O)_nH$$

where $R_1$ is lauric, myristic, cetyl, stearic, or oleic acid, or their derivatives and n = 2-10;

polyoxyethylene fatty acid ethers, having the formula

$$R_2\text{-}CO(C_2H_4O)_mH$$

where $R_2$ is lauric, myristic, or cetyl acids or their derivatives, single or double unsaturated octadecyl acids or their derivative, or double unsaturated eicodienoic acids or their derivatives and m ranges from 2-4;

diethanolamines, having the formula

$$(HOCH_2-CH_2)_2NCO-R_3$$

where $R_3$ is caprylic, lauric, myristic or linoleic acids or their derivatives;

long chain acyl hexosamides having the formula

$$R_4-NOCO-(CH_2)_b-CH_3$$

where b ranges from 10-18 and $R_4$ is a sugar molecule selected from a group consisting of glucosamine, galactosamine, and N-methylglucamine;

long chain acyl amino acid amides having the formula

$$R_5-CHC00H-NOC-(CH_2)_c-CH_3$$

where c ranges from 10-18 and $R_5$ is an amino acid side chain;

long chain acyl amides having the formula

$$HOOC-(CH_2)_d-N(CH_3)_2-(CH_2)_3-NCO-R_6$$

where $R_6$ is an acyl chain having 12-20 carbons and not more than two unsaturations, and d ranges from 1-3;

polyoxyethylene (20) sorbitan mono- or trioleate;

polyoxyethylene glyceryl monostearate or monooleate with 1-10 polyoxyethylene groups;

and glycerol monostearate or monooleate.

The surfactants can also be selected from a group consisting of polyoxyethylene (n) cetyl ethers or polyoxyethylene (n') cetyl amines, where n and n' each range from 5-10, polyoxyethylene (x, y, or z) stearyl, oleyl or linoleyl ethers, each having 2-10 polyoxyethylene units per acyl chain (x, y, and z range from 2-10), polyoxyethylene (x', y', or z') stearyl, oleyl, or linoleyl amines having 5-10 polyoxyethylene units per acyl chain (x', y', and z' range from 5-10, of polyoxyethylene (s) eicosamonoenoyl or polyoxyethylene (t) eicosadienoyl ethers where s and t ranges from 2-10, the corresponding amines, polyoxyethylene (s') eicosamonoenoyl and polyoxyethylene (t') eicosadienoyl amines, having 5-10 polyoxyethylene units per acyl group (s' and t' range from 5-10), and other 20 carbon acyl polyoxyethylene derivatives, either ethers or amines, having differing amounts of unsaturation depending on the specific location of the double bonds.

The lipophilic phase consisting of the structural lipids, structural steroids, and any other

lipophilic materials is blended with an aqueous phase consisting of an aqueous buffer and any aqueous soluble materials to be encapsulated, under shear mixing conditions, to form the paucilamellar lipid vesicles. "Shear mixing" is defined as the mixing of the lipophilic phase with the aqueous phase under turbulent or shear conditions which provide adequate mixing to hydrate the lipid and form lipid vesicles. The pump speeds are modified depending on the viscosity of the materials and the size of the orifices selected. "Shear mixing" is achieved by liquid shear which is substantially equivalent to a relative flow rate for the combined phases of about 5-30 m/s through a 1 mm radius orifice.

The invention also can be used for paucilamellar lipid vesicles having oil-soluble or oil-suspendable materials encapsulated therein. These vesicles are made by a procedure commencing with dispersing the material to be encapsulated in an oil or wax forming an oily phase. The oil or wax is a water immiscible oily solution selected from a group consisting of oils, waxes, natural and synthetic triglycerides, acyl esters, and petroleum derivatives, and their analogues and derivatives. The terms "disperse" or "dispersion" as used herein include dissolving or forming a suspension or colloid to yield a flowable phase. The oily phase containing the oil-dispersible material is mixed with the lipid phase and the combined oil-lipid phase is blended under shear mixing conditions with the aqueous phase. Surfactants useful in the encapsulation process are the same as those used to make

paucilamellar lipid vesicles with an aqueous core. These vesicles can then be used in the coupling process described herein.

In preferred embodiments of the invention, charge producing materials and steroids such as thiocholesterol are used in the formation of the paucilamellar lipid vesicles. Preferred negative charge producing materials are dicetyl phosphate, cetyl sulphate, phosphatidic acid, phosphatidyl serine, oleic acid, palmitic acid, or mixtures thereof. In order to provide a net positive charge to the vesicles, long chain amines, e.g., stearyl amines or oleyl amines, long chain pyridinium compounds, e.g., cetyl pyridinium chloride, quaternary ammonium compounds, or mixtures of these can be used. In addition, classic phospholipid materials may also be used for multilamellar lipid vesicles.

As noted, in addition to other uses, vesicles made using the methods disclosed herein can be used in diagnostic testing, e.g., agglutination testing for immunological systems. The vesicles can also be used as markers or labels for visualization, e.g., swelling or shrinking in the presence of an immune reaction, or for radiography or NMR.

Hydrophilic materials which can be encapsulated in the vesicles include macromolecules, viruses, immunological adjuvants such as muramyl dipeptide, peptide hormones such as insulin, calcitonin and glucagon, hypothalmic peptides,

pituitary hormones, growth factors such as angiogenic, epithelial and epidermal growth factors, lymphokines such as interleukin-2 and interferon, blood proteins such as hemoglobin and Factor VIII, water-soluble plant hormones and pesticides, radionucleotides, contrast materials for radiological and NMR diagnosis, cancer cytostatics, and antibiotics. Examples of lipophilic materials which can be encapsulated include steroid hormones, pheromones, porphyrins, organic pesticides, fungicides, insect repellants, and lipophilic vitamins and derivatives. Oil based materials include some additional lipophilic materials and materials which form colloids or suspensions in oil. A more complete listing of the types of pharmaceuticals that could be encapsulated in lipid vesicles is included in Gregoriadis, G., ed. Liposome Technology (CRC, Boca Raton, Fl.), Vols. 1-3 (1984).

The paucilamellar lipid vesicles can be made by a variety of devices which provides sufficient shear for shear mixing. There are a large variety of these devices available on the market including a microfluidizer such as is made by Biotechnology Development Corporation, a "French"-type press, or some other device which provides a high enough shear force and the ability to handle heated, semiviscous lipids. If a very high shear device is used, it may be possible to microemulsify powdered lipids, under pressure, at a temperature below their normal melting points and still form the lipid vesicles of the present invention.

A device which is particularly useful for making the lipid vesicles of the present invention has been developed by Micro Vesicular Systems, Inc., Vineland, New Jersey and is further described in United States Patent Application Serial No. 163,806, filed March 3, 1988. Briefly, this device has a substantially cylindrical mixing chamber with at least one tangentially located inlet orifice. One or more orifices lead to a reservoir for the lipophilic phase, mixed with an oil phase if lipid-core PLV's are to be formed, and at least one of the other orifices is attached to a reservoir for the aqueous phase. The different phases are driven into the cylindrical chamber through pumps, e.g., positive displacement pumps, and intersect in such a manner as to form a turbulent flow within the chamber. The paucilamellar lipid vesicles form rapidly, e.g., less than 1 second, and are removed from the chamber through an axially located discharge orifice. In a preferred embodiment, there are four tangentially located inlet orifices and the lipid and aqueous phases are drawn from reservoirs, through positive displacement pumps, to alternating orifices. The fluid stream through the tangential orifices is guided in a spiral flow path from each inlet or injection orifice to the discharge orifice. The flow paths are controlled by the orientation or placement of the inlet or injection orifices so as to create a mixing zone by the intersection of the streams of liquid. The pump speeds, as well as the orifice and feed line diameters, are selected to achieve proper shear mixing for lipid vesicle formation. As noted,

in most circumstances, turbulent flow is selected to provide adequate mixing.

No matter what device is used to form the paucilamellar lipid vesicles, if proper shear mixing is achieved they have a large, unstructured amorphous center surrounded by a plurality of lipid bilayers having aqueous layers interspersed there between. About four lipid bilayers is standard with 2-10 possible. The amorphous center may be entirely filled with an aqueous material, e.g., a buffer and any aqueous material to be encapsulated, or may be partially or totally filled with an oily material, forming lipid-core PLV's. If an aqueous center is used, the paucilamellar lipid vesicles will normally range in diameter from about 0.5-2 m while if an oily center is used, the size may increase to up to about 15-20 m depending upon the amount of oil used.

The following nonlimiting examples will further explain and illustrate the methods and procedures of the invention.

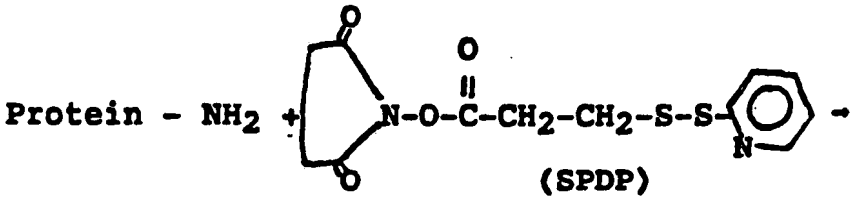
Example 1. Lectin Modification

A. General Reactions.

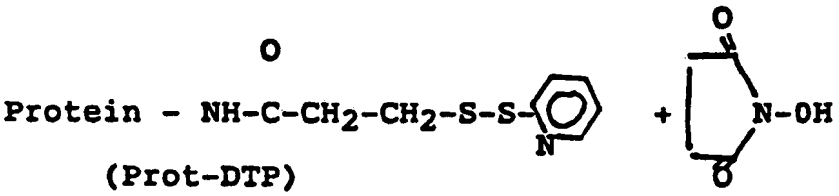
In this Example, two lectins, concanavalin A ("Con A") and wheat germ agglutinin ("WGA"), were modified using a preferred bifunctional coupling agent, N-hydroxysuccinimidyl 3-(2-pyridyldithio)

propionate ("SPDP"). The basic reactions involved are as follows:

I.

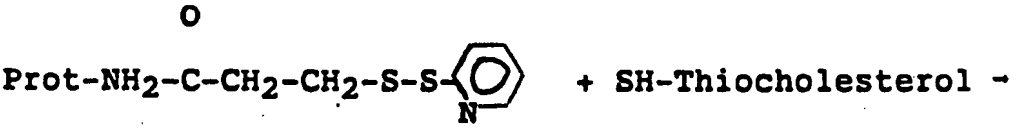


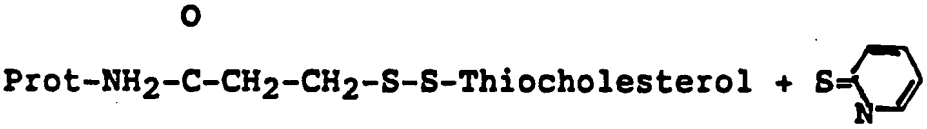
(SPDP)



(Prot-DTP)

II.





Once the SPDP is bound to the protein, forming a protein-dithiopropionate compound ("Prot-DTP"), the Prot-DTP can be coupled to the lipid vesicles containing a sulfhydryl group. The preferred lipid vesicles are paucilamellar lipid vesicles having a portion of the cholesterol used in forming the lipid vesicle replaced by thiocholesterol, yielding free SH groups. These free SH groups are coupled to the Prot-DTP by a covalent S-S bond. The number of protein molecules bound per lipid vesicle

can be modified by changing the number SPDP molecules bound to each molecule of protein, changing the amount of substitute protein (Prot-DTP) in contact with the lipid vesicles, and changing the percentage of the thiocholesterol in the lipid vesicles.

B. Concanavalin A-SPDP Bonding.

In the first experiment, 0.01 mMoles of Con A was incubated with varying amounts of SPDP for thirty minutes at room temperature. Stock solutions, specifically twenty-five mM solutions of the Con A in phosphate buffered saline, pH 7.5, 40 mM SPDP in methanol, and 50 mM dithiothreitol ("DTT") in distilled water, were used. Unreacted SPDP was eliminated by gel filtration through a PD10 gel column equilibrated with phosphate buffered saline, pH 7.5. Fractions were collected and the Con A-DTP complex was found to be concentrated in the void volume.

The DTP to protein binding ratio was determined using the following procedure. Approximately 0.5 ml of phosphate buffered saline was added to each fraction (bringing each fraction to approximately 1.0 ml) and 100 ml of DTT was added. The absorbance of the resulting pyridin-2-thione was measured at 343 nm. Table 1 shows the results of these experiments.

TABLE 1

| Con A (moles) | SPDP (Vol ml) | SPDP (mmoles) | Ratio SPDP/ Con A | DTP bound/ Con A |
|---|---|---|---|---|
| 0.01 | 1.25 | 0.05 | 5 | 1.68 |
| 0.01 | 2.5 | 0.10 | 10 | 2.70 |
| 0.01 | 5.0 | 0.20 | 20 | 2.76, 4.2, 4.0 |
| 0.01 | 10.0 | 0.40 | 40 | 4.6 |
| 0.01 | 20.0 | 0.80 | 80 | 1.6 |

As can be seen from Table 1, changing the amounts of SPDP modifies the amount of DTP bound per mole of lectin. Values of excess of four are relatively easy to obtain.

C. WGA-SPDP Binding.

In this experiment, the same procedure was used to couple the WGA to SPDP as was used to couple the Con A in paragraph B, except a SPDP/WGA ratio of twenty was used. The same stock solutions were used except a 25 mM solution of WGA was substituted for the Con A. Upon assay with DTT using the procedure previously described, approximately twelve SPDP molecules were bound to each WGA molecule.

D. Forming a Fluorescent WGA Conjugate.

In this experiment, the WGA was replaced with a WGA-fluoroscein isothiocyanate conjugate (WGA-FITC) purchased from Sigma Chemical Company. The same

procedures were used to couple the SPDP bifunctional agent to the WGA-FITC as have been described for coupling the WGA itself. Upon DTT analysis, approximately ten DTP molecules were bound to each WGA-FITC molecule. The WGA-FITC has substantially the same reactivity as WGA.

Example 2. Coupling of Protein to Lipid Vesicles

A. Formation of Lipid Vesicles.

In this Example, paucilamellar lipid vesicles were made using a syringe method to hydrate the lipid. The lipophilic phase is placed in one syringe, the aqueous phase (buffer) in another syringe coupled to the first by a stopcock, and the material is blended and formed into vesicles by transfer from syringe to syringe.

Table 2 lists the materials used to form "control" and "sample" lipid vesicles. The control vesicles are substantially identical to the sample vesicles except that almost two-thirds of the cholesterol is replaced with thiocholesterol in the sample vesicles. The materials used other than cholesterol are Brij 52 (ICI Americas) (polyoxyethylene (2) cetyl ether) as the main structural component, and dicetyl phosphate ("DCP") as a charge-producing agent. The Brij 52 is blended with the cholesterol and/or thiocholesterol and the DCP at 50-60°C. and hydrated with approximately 1.9 ml of phosphate buffered saline for about two minutes.

TABLE 2

| Material | "Control" | | | "Sample" | | |
|---|---|---|---|---|---|---|
| | mg | mmoles | % | mg | mmoles | % |
| Brij 52 | 63 | 0.19 | 76 | 63 | 0.19 | 76 |
| Cholesterol | 22 | 0.058 | 23 | 8 | 0.02 | 8 |
| Thiocholesterol | | | | 15 | 0.038 | 15 |
| DCP | 0.7 | 0.003 | 1 | 0.7 | 0.002 | 1 |

Paucilamellar lipid vesicles with a diameter of approximately 584 nm were formed in both the control and sample reactions. The sample vesicles had free SH groups for protein coupling.

B. Coupling of Thiocholesterol Lipid Vesicles and WGA-FITC-DTP

The following procedure was used to couple the WGA-FITC-DTP to the thiocholesterol-containing lipid vesicles. The WGA-FITC-DTP was formed using the procedure previously described by reacting 27 mg of WGA-FITC with 40 mmoles of SPDP. Recovery of the WGA-FITC-DTP was 1.6 ml at a concentration of approximately 1.35 mg/ml.

Three different amounts of WGA-FITC-DTP were mixed with 200 ml of the thiocholesterol modified lipid vesicles (2.5 micromoles lipids) and kept overnight at room temperature. Unreacted WGA-FITC-DTP was removed by centrifugation on a discontinuous dextran gradient. The gradient was

prepared by mixing about 0.5 ml of the sample with 2 ml of 20% dextran and overlaying the sample successively with 2 ml of 15%, 10%, and 5% dextran. The dextran gradient containing the lipids was centrifuged for approximately 15 minutes at 3,500 rpm in a Beckman Table Top centrifuge. Unreacted lectins stay in the 20% layer while the lipid vesicles go to the top.

The fluorescence of the control and sample lipid vesicles were measured using an absorbance of 490 nm and an emission of 520 nm. The results, as compared with a WGA-FITC-DTP standard, are shown in Table 3. In all cases, a substantial binding to the lipid vesicles was observed. Three different amounts of WGA-FITC-DTP were used.

TABLE 3

| Assay | (A) | (B) | (C) |
|---|---|---|---|
| WGA-FITC (1.35 mg/ml) | 0.121 mg (90 ml) | 0.430 mg (320 ml) | 0.810 mg (600 ml) |
| Lipids (200 ml) | 2.5 mmol | 2.5 mmol | 2.5 mmol |
| WGA-FITC Bound | 1.46 mmol | 4.8 mmol | 7.92 mmol |
| WGA-FITC Bound/ Lipo | 7,800 | 26,000 | 42,000 |
| WGA-FITC Bound/ SH-Chol | 1/63 | 1/19 | 1/12 |

These nonlimiting Examples show the efficacy of the present invention. These Examples, and the description herein, may lead others to further obvious modifications of the present invention. Such other modifications are included within the following claims.

What is claimed is:

1. A method of coupling targeting molecules to lipid vesicles while retaining the targeting specificity of said targeting molecules comprising the steps of:

selecting a targeting molecule which has a free $NH_2$ group thereon,

reacting said targeting molecule with a bifunctional agent which reacts with said $NH_2$ group on said targeting molecule and which incorporates a free SH group onto said targeting molecule,

forming a lipid vesicle having a steroid with a free SH group as one of its structural components,

reacting said lipid vesicle having a free SH group thereon and said targeting molecule with said free SH group,

thereby coupling said targeting molecule to said lipid vesicle.

2. The method of claim 1 wherein said steroid with a free SH group is selected from a group consisting of thiocholesterol, its analogs, and its derivatives.

3. The method of claim 2 wherein said lipid vesicle comprises a nonphospholipid lipid vesicle.

4. The method of claim 3 wherein said nonphospholipid lipid vesicle comprises polyoxyethylene fatty esters, polyoxyethylene fatty acid ethers, diethanolamines, long-chain acyl amino acid amides, long-chain acyl amides, polyoxyethylene sorbitan mono and tristearates and oleates, polyoxyethylene glyceryl monostearates and monooleates, and glyceryl monostearates and monooleates.

5. The method of claim 4 wherein said nonphospholipid lipid vesicle comprises a paucilamellar lipid vesicle.

6. The method of claim 2 wherein said bifunctional agent is selected from a group consisting of N-hydroxysuccinimidyl 3-(2-pyridyethio)propionate, and its derivatives, and chemical analogs.

7. The method of claim 1 wherein said targeting molecule is selected from a group consisting of peptide hormones, and their derivatives and chemical analogs.

8. The method of claim 1 wherein said targeting molecule comprises a protein.

9. The method of claim 8 wherein said protein is selected from a group consisting of lectins, immunoglobulins, and their derivatives and chemical analogs.

10. The method of claim 9 wherein said lectin is selected from a group consisting of concanavalin A, wheat germ agglutinin, and their derivatives and chemical analogs.

11. The method of claim 9 wherein said immunoglobulin is selected from a group consisting of monoclonal antibodies, chimeric antibodies, and portions or fragments thereof.

12. The method of claim 5 wherein said nonphospholipid lipid vesicle further comprises a charge-producing agent as a structural component.

13. A method of delivering a specified material to a particular location or tissue in a human or animal body comprising the steps of:

encapsulating said specified material in a lipid vesicle, said lipid vesicle containing a steroid having a free SH group as one of its structural components,

selecting a proteinaceous targeting molecule which reacts with said particular location or tissue,

reacting said proteinaceous targeting molecule with a bifunctional agent that reacts with free $NH_2$ groups on said targeting molecule and provides a free SH group, thereby forming a modified targeting molecule,

reacting a plurality of said modified targeting molecules with said lipid vesicle so that at least one of said modified targeting molecules is bound to the surface of said lipid vesicle,

introducing said lipid vesicle having said modified targeting molecule bound thereto into said human or animal, and

allowing said targeting molecule to react with its target, thereby bringing said encapsulated material to said specified location or tissue.

14. The method of claim 13 wherein said lipid vesicle comprises a nonphospholipid lipid vesicle.

15. The method of claim 14 wherein said nonphospholipid lipid vesicle comprises a paucilamellar lipid vesicle.

16. The method of claim 13 wherein said proteinaceous targeting molecule is selected from a group consisting of immunoglobulins, their derivatives, and chemical analogs.

17. The method of claim 16 wherein said immunoglobulin is selected from a group consisting of monoclonal antibodies, chimeric antibodies, and fragments and portions thereof.

18. A lipid vesicle having a targeting molecule attached thereto comprising a lipid vesicle containing a steroid having free SH groups as one of

its structural components, said targeting molecule being attached to said lipid vesicle by an S-S bond between a free SH group on said targeting molecule and a free SH group on said lipid vesicle.

19. The lipid vesicle of claim 18 wherein said vesicle comprises a nonphospholipid lipid vesicle.

20. The lipid vesicle of claim 19 wherein said lipid vesicle comprises a paucilamellar lipid vesicle.

21. The lipid vesicle of claim 18 wherein said targeting molecule is selected from a group consisting of proteins, peptide hormones, and their derivatives and chemical analogs.

22. The lipid vesicles of claim 21 targeting molecule is a protein selected from a group consisting of lectins, immunoglobulins, monoclonal antibodies, chimeric antibodies, and their derivatives, fragments, and chemical analogs.

23. The lipid vesicles of claim 18 wherein said targeting molecule is attached to said lipid vesicle by a bifunctional agent attaching to a free $NH_2$ group on said targeting molecule and supplying a free SH group.

24. The lipid vesicles of claim 23 wherein said bifunctional agent comprises N-hydroxysuccinimidyl 3-(2-pyridyethio)propionate, and its derivatives, and chemical analogs.

25. The lipid vesicle of claim 18 wherein said vesicle has a diagnostic agent encapsulated therein.

26. The lipid vesicle of claim 25 wherein said targeting molecule comprises an immunoglobulin.

27. The lipid vesicle of claim 26 wherein said immunoglobulin is selected from a group consisting of monoclonal antibodies, chimeric antibodies, and portions and fragments thereof.

28. The lipid vesicle of claim 25 wherein said diagnostic agent is selected from a group consisting of immunodiagnostic agents, visualization agents, high density particles and magnetic particles.

# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00257

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL. (5): A61K 9/127,37/22; 39/44; B01J 13/02

U.S. CL. 264/4.3; 424/85.8, 420, 450; 428/402.2; 436/829; 514/963.

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| U.S. CL. | 264/4.3; 424/85.8, 420, 450; 428/402.2; 436/829; 514/963. |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category [10] | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| Y | EP, A, 0,144,084 ISHIMORI et al. 12 June 1985 see examples 1-12; and page 9, lines 12-15. | 1-28 |
| Y,P | US, A, 4,804,539 GUO et al. 14 February 1989 see col.8, line 43-col.9, line 68. | 1-28 |
| Y | US, A, 4,217,344 VANLERBERGHE 12 August 1980 See Ex. 1 and 2; and col.17, line 67-col.18, line 2. | 3-5,12,14,15, 19,20 |
| A | US, A, 4,429,008 MARTIN et al. 31 January 1984. | 1-28 |
| A | US, A, 4,603,044 GEHO et al. 29 July 1986. | 1-28 |
| A | US, A, 4,605,630 KUNG et al. 12 August 1986. | 1-28 |
| A,P | US, A, 4,855,090 WALLACH 08 August 1989. | 1-28 |

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 24 APRIL 1990 | 04 JUN 1990 |

| International Searching Authority | Signature of Authorized Officer |
|---|---|
| ISA/US | for RICHARD D. LOVERING Ngoc Ho Nguyen Ho NGUYEN |

Form PCT/ISA/210 (second sheet) (Rev.11-87)